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A mutation that allows endosperm development without fertilization

(reproduction/embryogenesis/seed/fruit/apomixis)

NIR OHAD, LINDA MARGOSSIAN, YUNG-CHAO HSU, CHAD WILLIAMS, PETER REPETTI, AND ROBERT L. FISCHER*

Department of Plant Biology, University of California, Berkeley, CA 94720-3102

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ABSTRACT **The mechanisms that initiate reproductive development after fertilization are not understood. Reproduction in higher plants is unique because it is initiated by two fertilization events in the haploid female gametophyte. One sperm nucleus fertilizes the egg to form the embryo. A second sperm nucleus fertilizes the central cell to form the endosperm, a unique tissue that supports the growth of the embryo. Fertilization also activates maternal tissue differentiation, the ovule integuments form the seed coat, and the ovary forms the fruit. To investigate mechanisms that initiate reproductive development, a female-gametophytic mutation termed *fie* (fertilization-independent endosperm) has been isolated in *Arabidopsis*. The *fie* mutation specifically affects the central cell, allowing for replication of the central cell nucleus and endosperm development without fertilization. The *fie* mutation does not appear to affect the egg cell, suggesting that the processes that control the initiation of embryogenesis and endosperm development are different. *FIE/fie* seed coat and fruit undergo fertilization-independent differentiation, which shows that the *fie* female gametophyte is the source of signals that activates sporophytic fruit and seed coat development. The mutant *fie* allele is not transmitted by the female gametophyte. Inheritance of the mutant *fie* allele by the female gametophyte results in embryo abortion, even when the pollen bears the wild-type *FIE* allele. Thus, *FIE* carries out a novel, essential function for female reproductive development.**

A fundamental problem in biology is to understand how fertilization initiates reproductive development. As shown in Fig. 1, in higher plants, the ovule generates the female gametophyte, which is composed of egg, central, synergid, and antipodal cells (1). All are haploid except the central cell, which contains two daughter nuclei that fuse before fertilization. One sperm nucleus fertilizes the egg to form the zygote, whereas another sperm nucleus fuses with the diploid central cell nucleus to form the triploid endosperm nucleus (2). The two fertilization products undergo distinct patterns of development. In *Arabidopsis*, the embryo passes through a series of stages that have been defined morphologically as preglobular, globular, heart, cotyledon, and maturation (3, 4). The primary endosperm nucleus undergoes a series of mitotic divisions to produce nuclei that migrate into the expanding central cell (5, 6). Cytokinesis sequesters endosperm cytoplasm and nuclei into discrete cells (7) that produce storage proteins, starch, and lipids that support embryo growth (8). Fertilization also activates development of the integument cell layers (Fig. 1) of the ovule that become the seed coat and induces the ovary (Fig. 1) to grow and form the fruit, or silique, in *Arabidopsis*. Little is known about the mechanisms that control egg and central cell differentiation or about the genetic pathways that activate reproductive development in response to fertilization. To address these issues, we generated and analyzed mutant *Arabidopsis* plants that undergo several reproductive processes in the absence of fertilization.

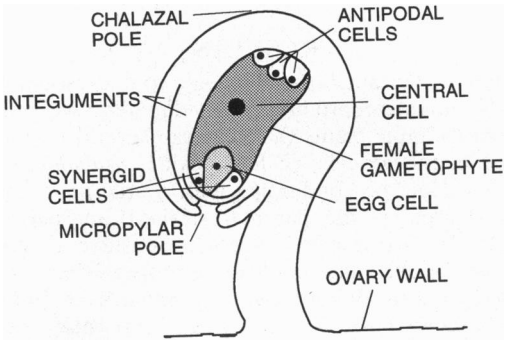

FIG. 1. Schematic representation of an ovule and female gametophyte. Nuclei are represented by black circles.

MATERIALS AND METHODS

Growth and Phenotype of Plants. Plants were grown under low humidity conditions (<50%) in glass houses under 16-hr light/8-hr dark photoperiods generated by supplemental lighting. Plants were grown at high humidity (>80%) in a lighted incubator (Percival, Boone, IA). To test for fertilization-independent development, flower buds from plants that had not yet begun to shed pollen [stage 12 (9)] were opened, immature anthers were removed, and the flower bud was covered with a plastic bag. Seven days later, the silique was measured and dissected, and the number of seed-like structures and degenerating ovules were counted. To determine the frequency of seed abortion following fertilization, siliques were harvested 10 days after self-pollination and dissected, and wild-type and aborted seeds were counted.

Genetic Mapping. Heterozygous *FIE/fie* (Landsberg erecta ecotype; *fie* stands for fertilization-independent endosperm) plants were crossed as males with female plants (Columbia ecotype) that were homozygous for glabrous 1 (*gl1*), a recessive mutation on chromosome 3 (10) that prevents trichome formation (11). Because the mutant *fie* allele is only transmitted through the male gametophyte, *FIE/fie* progeny were crossed as males a second time to female *gl1/gl1* (Columbia ecotype) plants. Fifty-five progeny were scored for the segregation of the wild-type *FIE* and mutant *fie* alleles, for the presence of trichomes, and for alleles of molecular markers as described (12). This analysis indicated that *fie* is 21.2 ± 6.4 centimorgans (cM) above *GL1* and 13.3 ± 6.0 cM below the molecular marker nga162 on chromosome 3. Genetic recombination frequencies and map distances were calculated according to Koornneef and Stam (13) and Kosambi (14).

Light Microscopy. Nomarski photographs of whole-mount embryos and endosperm were obtained by fixing longitudinally

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Abbreviation: GUS, β-glucuronidase.

*To whom reprint requests should be addressed. e-mail: rfischer@mendel.berkeley.edu.

slit siliques in an ethanol/acetic acid (9:1) solution overnight, followed by two washes in 90% and 70% ethanol, respectively. Siliques were cleared with a chloral hydrate/glycerol/water solution (8:1:2, wt/vol) (15). Whole mount preparations were fixed and stained with hematoxylin (16). Embryo and endosperm were photographed with a Zeiss Axioskop microscope by using Nomarski optics that permits visualization of optical sections within the seed.

β-Glucuronidase (GUS) Histochemical Assays. GUS activity was detected histochemically as described (17).

Image Processing. Photographs were scanned using a Microtek scanner. Pictures were processed for publication using Adobe Photoshop 3.0 and printed on a Tektronix Phaser 400 color printer.

RESULTS

Isolation of Mutant Lines. To begin to understand mechanisms that initiate reproductive development, we generated mutant *Arabidopsis* plants that undergo several reproductive processes in the absence of fertilization. *Arabidopsis* plants homozygous for the conditional male sterile *pop1* mutation (18) were used as the parental strain (Landsberg erecta ecotype). Fertility in *pop1* plants is sensitive to humidity because *pop1* pollen does not hydrate properly due to a defect in wax biosynthesis. When grown at permissive condition [high relative humidity (>80%)], *pop1* plants were male fertile and produced long siliques (Fig. 2*A*) with many viable seeds (Fig. 2*D*). By contrast, when grown at nonpermissive conditions [low relative humidity (<50%)], *pop1* plants were male sterile and produced short siliques (Fig. 2*B*) with no seeds (Fig. 2*E*). Thus, silique elongation is a marker for reproductive events.

To isolate mutations, homozygous *pop1* seeds were mutagenized with ethylmethanesulfonate and ≈50,000 M1 plants were screened for silique elongation at nonpermissive conditions. Rare M1 plants were identified that displayed heterozygous sectors with elongated siliques (data not shown). These plants were transferred to permissive conditions to ensure the production of viable M2 seed. Plants from M2 and M3 families grown at nonpermissive conditions were rechecked for nonsectored silique elongation. To eliminate any effects of the *pop1* mutation or other ethylmethanesulfonate-induced lesions on the mutant phenotype mutant plants were backcrossed twice, as males, to wild-type plants. After removing the *pop1* mutation, fertilization-independent phenotypes were confirmed after manual removal of anthers from immature flowers before pollen was shed. A total of 12 lines were identified that displayed elongated siliques in the absence of fertilization (data not shown).

FIG. 2. Silique and seed development in wild-type and mutant *Arabidopsis* plants. (*A* and *D*) *pop1/pop1* silique grown at high humidity. Average silique length was 12 ± 1 mm (48 siliques measured). (*B* and *E*) *pop1/pop1* silique grown at low humidity. Average silique length was 3.2 ± 0.3 mm (25 siliques measured). Arrow in *E* points to an unfertilized ovule. (*C* and *F*) *FIE/fie* and *pop1/pop1* silique grown at low humidity. Anthers were removed before anthesis as described. Average silique length was 5.2 ± 1.0 mm (21 siliques measured). Arrow in *F* points to a seed-like structure. (Bar = 1 mm for *A–C*; bar = 0.33 mm for *D–F*.)

Fertilization-Independent Endosperm, Seed Coat and Silique Development. In a representative line chosen for further study, heterozygous plants produced by back crosses to wild-type plants generated elongated siliques after anther removal (Fig. 2*C*) with numerous seed-like structures (Fig. 2*F*). These results indicated that heterozygous mutant plants were capable of silique elongation and seed-like structure development in the absence of fertilization.

We compared the development of the mutant seed-like structures to that of wild-type seeds. Fig. 3*A* shows a mature, unfertilized wild-type ovule and female gametophyte. After fertilization, the endosperm nucleus replicated (Fig. 3*B*) and daughter nuclei migrated into the expanding central cell (Fig. 3*C*). Ultimately, a syncytium of endosperm nuclei was produced (Fig. 3*G*). Nuclear divisions of the endosperm preceded (Fig. 3 *B* and *C*) the zygotic divisions that formed the globular stage embryo (Fig. 3*G*). Embryo, endosperm or seed coat development did not occur in wild-type plants in the absence of fertilization (Fig. 2*E*).

Development of the ovule and female gametophyte in heterozygous mutant plants was normal (data not shown). Just before flower opening, female gametophytes in these plants contained a single, prominent central cell nucleus (Fig. 3*D*). Subsequently, in the absence of fertilization, central cells with two large nuclei were detected (Fig. 3*E*). Further divisions resulted in the production of additional nuclei that migrated into the expanded central cell (Fig. 3*F*). Later in development, a nuclear syncytium was formed with abundant endosperm nuclei (Fig. 3*H*). These results indicated that the central cell in mutant female gametophytes initiated endosperm development in the absence of fertilization. We have named this mutation *fie* for fertilization-independent endosperm. By contrast, replication of other nuclei in *fie* female gametophytes (egg, synergid, or antipodal) was not detected (Fig. 3). Thus, the *fie* mutation specifically affects replication of the central cell nucleus.

We analyzed the frequency of multinucleate central cell formation in *fie* female gametophytes by comparing the percentage of multinucleate central cells at 3, 5, and 6 days after emasculation of heterozygous *FIE/fie* and control wild-type flowers. As shown in Fig. 4, at each time point only 3–5% of wild-type central cells had more than one nucleus. Because none had more than two nuclei, most likely these represented central cells with haploid nuclei that had not fused during female gametophyte development. By contrast, the percentage of central cells in female gametophytes from *FIE/fie* siliques with two or more nuclei increased from 21% to 47% over the same time period. These results indicated that the *fie* mutation caused a significant increase in formation of multinucleate central cells in the absence of fertilization. The fact that close to 50% of the female gametophytes in heterozygous plants had multinucleate central cells suggested that *fie* is a gametophytic mutation because a 1:1 segregation of wild-type and mutant *fie* alleles occurs during meiosis.

We compared the fertilization-independent development of the maternal seed coat in *FIE/fie* seed-like structures to that of fertilized wild-type seeds. The seed coat in wild-type *Arabidopsis* (Fig. 3*G*) is generated by the integuments of the ovule (Fig. 3*A*) and surrounds the developing embryo and endosperm. Similarly, *FIE/fie* ovule integuments (Fig. 3*D*) formed a seed coat (Fig. 3*H*) that surrounded the developing mutant endosperm. These results indicated that the *fie* mutation activated both endosperm development and maternal sporophytic seed coat (Fig. 3*H*) and silique (Fig. 2*F*) differentiation that support reproduction. No other effects on

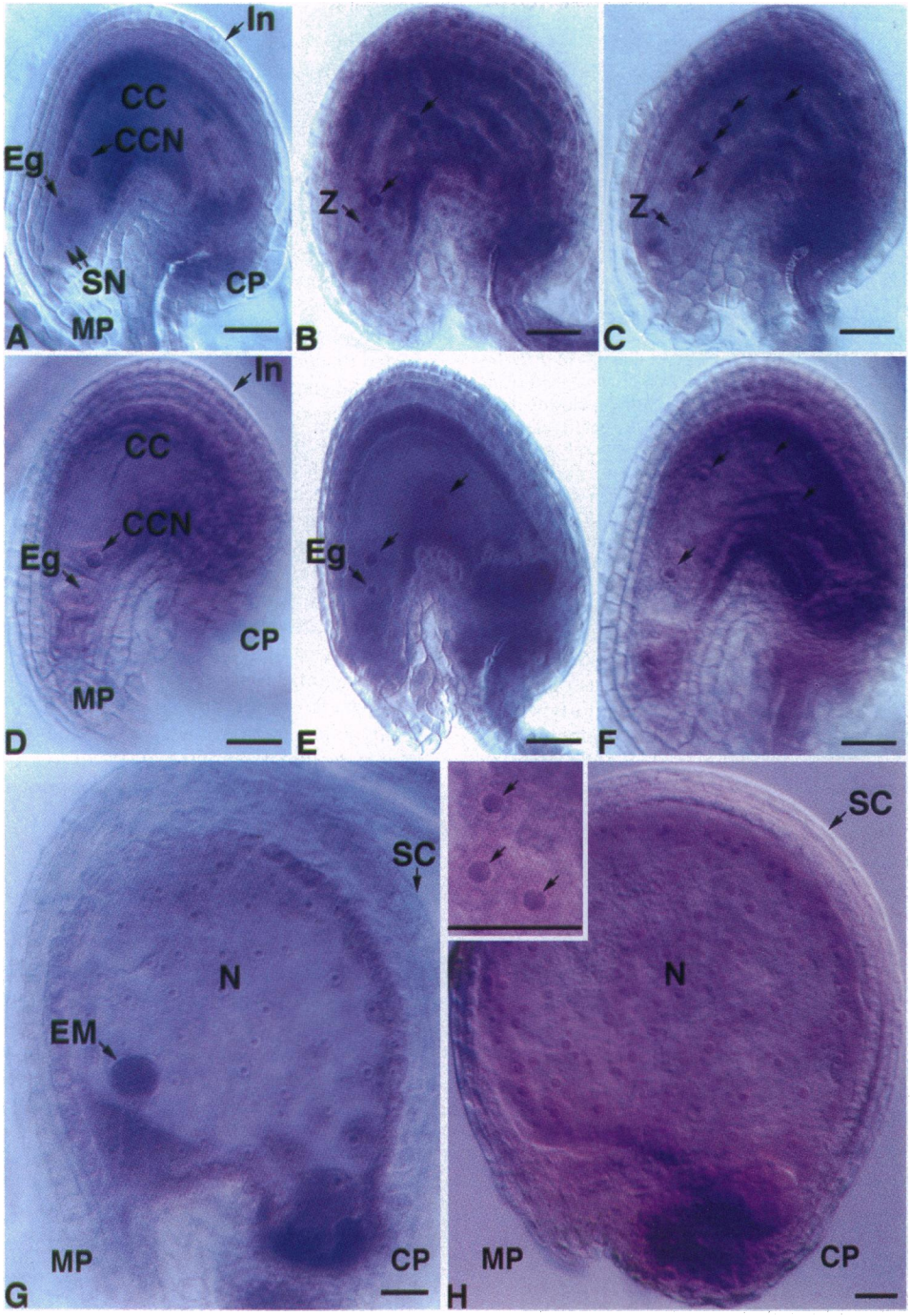

FIG. 3. Embryo, endosperm, and seed coat development in wild-type and mutant *Arabidopsis* plants. (*A–C* and *G*) Seeds from wild-type plants. (*A*) Unfertilized ovule and female gametophyte from flower just before flower opening. (*B* and *C*) Fertilized seed from self-pollinated flower immediately after flower opening. (*G*) Seed with globular embryo from flower 2 days after self-pollination. (*D–F* and *H*) Seed-like structures from emasculated heterozygous *FIE/fie* plants. Immature anthers were removed before pollen was shed, and the flower bud was covered with a plastic bag. (*D*) Unfertilized ovule and female gametophyte. (*E* and *F*) Seed-like structures 3 days after anther removal. (*H*) Seed-like structure 7 days after anther removal. CC, central cell; CCN, central cell nucleus; CP, chalazal pole; Eg, egg nucleus; EM, embryo; N, endosperm; In, integuments; MP, micropylar pole; SC, seed coat; SN, synergid cell nucleus; Z, zygote. Unlabeled arrows indicate endosperm nuclei. (Bars = 25 μm.)

sporophytic growth and development were detected in *FIE/fie* plants (data not shown).

The *fie* Mutant Allele Is Not Transmitted by the Female Gametophyte to the Next Generation. To understand the mode of inheritance of the *fie* mutation, we analyzed the progeny of reciprocal crosses. *FIE/fie* females, crossed to wild-type males, produced siliques (Fig. 5*A*) with approximately equal numbers of viable seeds with normal green embryos (Fig. 5*C*) and nonviable white seeds (Fig. 5*D*) with embryos aborted at the heart stage (344:375, 1:1, $\chi^2 = 1.3, P > 0.2$). Viable seeds from this cross were germinated and all 120 F1 progeny generated were wild type. That is, none of the F1 progeny had significant levels of F2 aborted seeds in their siliques after self-pollination. Nor did the F1 progeny demonstrate fertilization-independent development. This indicated that the presence of the *fie* mutant allele in the female gametophyte, even when the male provided a wild-type allele, resulted in embryo abortion. Thus, the *fie* mutation is not transmitted by the female gametophyte to the next generation.

To study transmission of *fie* through the male gametophyte, we pollinated female wild-type plants with pollen from male

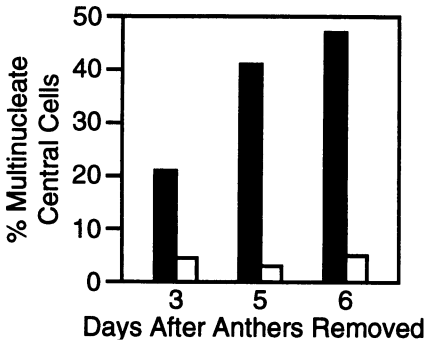

FIG. 4. Accumulation of multinucleate central cells in *FIE/fie* siliques. Immature anthers were removed before pollen was shed from *FIE/fie* flower buds (closed bars) and wild-type control flower buds (open bars). At the indicated times, siliques were dissected, cleared, fixed, and stained as described. Female gametophytes were visualized with Nomarski optics, and the number of central cell nuclei were counted. For wild type, the percentage of multicellular nuclei was 5% (588 checked) at 3 days, 3% (584 checked) at 5 days, and 5% (576 checked) at 6 days after anther removal. For *FIE/fie*, the percentage of multicellular nuclei was 21% (972 checked) at 3 days, 41% (646 checked) at 5 days, and 47% (828 checked) at 6 days after anther removal.

FIE/fie plants. Siliques from these crosses contained no aborted F1 seed (Fig. 5*B*). F1 plants were examined and a 1:1 segregation of wild-type and *FIE/fie* genotype was observed (62:58, $\chi^2 = 0.13$, $P > 0.5$). This indicated that wild-type and mutant *fie* alleles were transmitted by the male gametophyte with equal efficiency. That is, *fie* does not affect male gametophyte, or pollen grain, function.

Results from reciprocal crosses were verified by analyzing the progeny from self-pollinated *FIE/fie* plants. Self-pollinated siliques displayed 1:1 segregation of normal and aborted seeds (282:286, $\chi^2 = 0.03$, $P > 0.8$). Viable seed from self-pollinated siliques were germinated and a 1:1 (71:64, $\chi^2 = 0.36$, $P > 0.5$) segregation of wild-type and *FIE/fie* progeny was observed. These results confirmed that inheritance of a *fie* mutant allele by the female gametophyte resulted in embryo abortion and that inheritance of a *fie* mutant allele by the male gametophyte did not affect pollen function. Thus, the wild-type *FIE* allele probably carries out a function unique to the female gametophyte and does not appear to be needed for male fertility.

In the genetic analysis described above, all plants that displayed fertilization-independent development also produced 50% aborted seeds after self-pollination. The fact that perfect cosegregation was observed among 372 plants tested suggests that a single *fie* locus is responsible for both mutant phenotypes. This conclusion is supported by the fact that 11 other *fie* lines, independently isolated in our screen, also displayed both phenotypes (data not shown). It is not possible to test for allelism by genetic complementation because these mutations are gametophytic. However, preliminary mapping experiments suggested that *fie* (see *Material and Methods*) and an additional three lines (data not shown) all map to the same region on chromosome 3, and may represent multiple *fie* alleles.

What is the relationship among the two *fie* phenotypes, fertilization-independent endosperm development, and the production of aborted embryos after pollination? One possibility is that premature replication of the central cell nucleus in the *fie* female gametophyte prevents fusion of the male and central cell nuclei, resulting in an endosperm that lacks a set of paternal chromosomes. Deviation from the 2:1 maternal/paternal ratio of chromosomes in the endosperm has been shown in certain plant species to result in defective endosperm formation and embryo abortion (19). To address this issue, we performed genetic crosses using genetically marked pollen from plants homozygous for a seed-specific reporter gene [β-conglycinin promoter (20) fused to GUS coding sequences (21)]. Control crosses to wild-type females generated seeds that displayed GUS activity in the endosperm and embryo (Fig. 6*A*). When *FIE/fie* females were used in genetic crosses, GUS activity encoded by the paternal marker gene was observed in the endosperm and aborted embryo (Fig. 6*B*). Similar results were observed when endosperm and embryo were separated before staining (data not shown) and when promoters from other genes expressed during seed development, such as DC8 (22) and lipid transfer protein (23), were used (data not shown). No staining was detected in control experiments when pollen from nontransgenic plants were used (data not shown). These results indicated that both the *fie* mutant egg and central cell received a paternal set of chromosomes and activated transcription of seed-specific paternal genes.

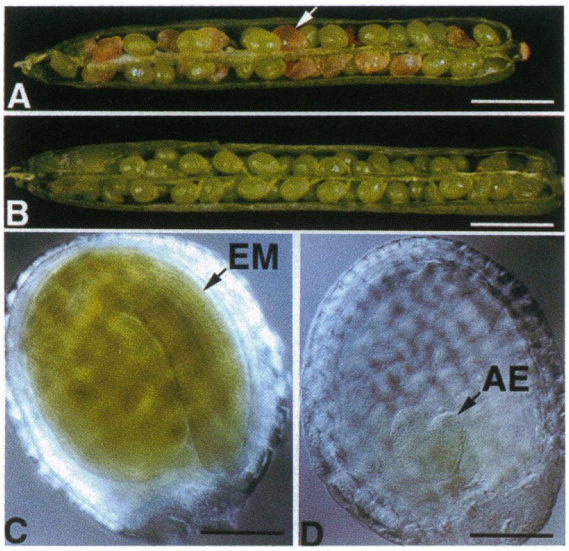

FIG. 5. Inheritance of the *fie* mutant allele by the female gametophyte results in embryo abortion. (*A*) Silique obtained from a *FIE/fie* female pollinated with wild-type pollen. White arrow points to an aborted seed. (*B*) Silique obtained from a female wild-type plant pollinated with pollen from a *FIE/fie* plant. (*C*) Viable green seed obtained from silique in *A*. (*D*) Defective white seed from silique in *A*. AE, aborted embryo; SC, seed coat; EM, embryo; N, endosperm. (Bar = 2 mm for *A* and *B*; bar = 150 μm for *C* and *D*.)

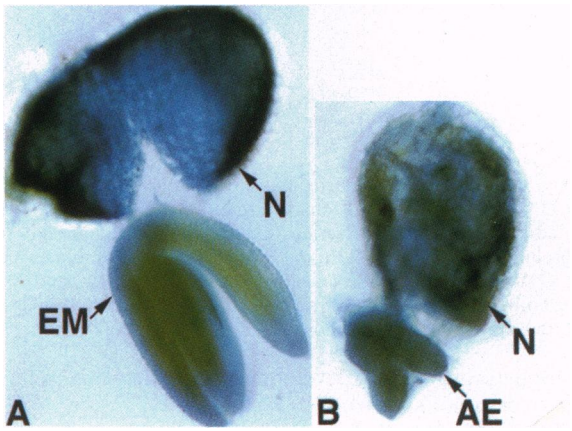

FIG. 6. Double fertilization takes place in the *fie* female gametophyte. Pollen from a wild-type plant homozygous for a chimeric reporter gene consisting of the promoter for an α′ subunit of β-conglycinin gene (20) fused to GUS coding sequences (21). Seven days after pollination, seeds were dissected and stained for GUS activity. (*A*) Embryo and endosperm produced when a wild-type female plant was used. (*B*) Endosperm and aborted embryo when a female *FIE/fie* heterozygote was used. AE, aborted embryo; EM, embryo; N, endosperm.

DISCUSSION

In wild-type plants, fertilization initiates embryogenesis and endosperm formation and activates maternal seed coat and silique development. The results presented here indicate that specific aspects of plant reproductive development can occur in *FIE/fie* plants in the absence of fertilization. These include silique elongation, seed coat formation, and endosperm development. Morphological analysis shows that early aspects of fertilization-independent *fie* endosperm development closely resemble fertilized wild-type endosperm development (Fig. 3). First, the *fie* central cell nucleus is stimulated to undergo replication. Second, nuclei that are produced migrate from the micropylar end of the central cell and take up new positions in the central cell. Third, the developing *fie* central cell expands to form an endosperm cavity. Thus, the requirement for fertilization to initiate these early events in endosperm formation has been eliminated by the *fie* mutation. This suggests that *FIE* plays a role in a signal transduction pathway that links fertilization with the onset of central cell nuclear replication and early endosperm development.

Mechanisms for Regulation of Endosperm Development by *FIE*. One can envision two possible mechanisms for how *FIE* regulates replication of the central cell nucleus in response to fertilization. As shown in Fig. 7*A*, the protein encoded by the *FIE* gene may be involved in a positive regulatory interaction. In this model, *FIE* is required for the central cell to initiate endosperm development. Normally, fertilization is needed for the presence of active FIE protein. The *fie* mutation results in the presence of active protein in the absence of fertilization. Alternatively, as shown in Fig. 7*B*, *FIE* may by involved in a negative regulatory interaction. In this model, the function of FIE protein is to prevent the central cell from initiating endosperm development, and fertilization results in the inactivation of FIE protein. The *fie* mutation results in the production of inactive protein, so that fertilization is no longer required to initiate endosperm development. Recently, it has been shown that cyclin-dependent kinase complexes, related to those that function in mammals, control the induction of DNA synthesis and mitosis in maize endosperm (24). Because *fie* stimulates replication of the central cell, *fie* may, either directly or indirectly, impinge upon cell cycle control of the central cell nucleus, allowing replication to take place in the absence of fertilization.

The *fie* Mutation Uncouples the Initiation of Endosperm and Embryo Development. In the absence of fertilization, the effect of the *fie* mutation on egg and central cell development differs dramatically. Whereas the *fie* central cell initiates endosperm development, the *fie* egg does not initiate embryogenesis. Thus, events that control the initiation of embryo and endosperm must be different. This conclusion is consistent with the observation that in wild-type *Arabidopsis* reproduction, the free-nuclear division of the endosperm is initiated before the first zygotic division (Fig. 3*B*; ref. 5). Studies on the evolution of flowering plants from their nonflowering ancestors have provided clues about the relationship between embryo and endosperm development. It has been proposed that endosperm is a modification of a supernumerary embryo into a specialized tissue dedicated to the nourishment of its genetically identical sister embryo (25). If this model is correct, then our results suggest that during evolution the genetic pathways for the initiation of embryo and endosperm development have diverged.

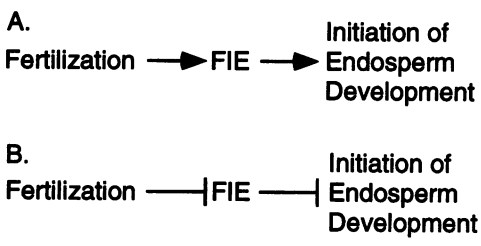

FIG. 7. Models for the induction of endosperm development in *Arabidopsis*. Schematic diagrams are shown for the regulatory hierarchy controlling the initiation of endosperm development. (*A*) Arrows indicate a positive regulatory interaction. (*B*) Bars indicate a negative regulatory interaction.

Mechanisms for *fie*-Induced Embryo Abortion. In addition to affecting fertilization-independent development, *fie* is also a gametophytic embryo lethal mutation. Inheritance of the mutant *fie* allele by a female gametophyte results in embryo abortion, even when the pollen bears the wild-type allele. Because *fie* is a female gametophytic mutation, it is distinct from zygotic embryo/endosperm lethal mutations that have been isolated previously in *Arabidopsis* (26, 27) and maize (28) where embryo abortion is due to the inheritance of both maternal and paternal recessive defective alleles. Why is the mutant *fie* allele not transmitted by the female gametophyte? One possibility is that presence of a mutant *fie* allele in the female gametophyte results in an egg, central cell, or both, that do not function or interact properly after fertilization. Alternatively, gene dosage (29) or imprinting (30) could play a role. That is, a single wild-type paternal *FIE* gene, or a wild-type paternal *FIE* gene that has been silenced during development of male gametes, may not be able to rescue two mutant *fie* alleles contributed by the maternal parent, thus resulting in embryo abortion.

Communication Between the *fie* Female Gametophyte and the Sporophytic Ovule and Carpels. The analysis of *FIE/fie* mutant plants has provided clues about interactions between endosperm and maternal sporophytic tissues. *FIE/fie* ovule integuments surrounding a mutant *fie* female gametophyte initiate seed coat development (Figs. 2*F* and 3*H*), whereas *FIE/fie* integuments in contact with a quiescent wild-type female gametophyte do not develop. This suggests that the *FIE/fie* ovule integuments initiate seed coat differentiation in response to a signal produced by the *fie* female gametophyte. We propose that the source of the signal is the mutant *fie* central cell that has initiated endosperm development, although we cannot rule out the participation of other cells in the *fie* female gametophyte. In wild-type plants, most likely, fertilization of the central cell produces an endosperm that activates seed coat development. This is consistent with experiments showing that the maize endosperm interacts with nearby maternal cells (31). *FIE/fie* plants also display fertilization-independent elongation of the ovary to form the silique. We propose that a signal is produced by the developing seed-like structures to initiate silique elongation. This is in agreement with experiments suggesting that seeds are the source of hormones, auxins and gibberellins, that activate fruit development (32). Taken together, these results suggest that the fertilized female gametophyte activates maternal developmental programs.

Relationship Between *fie* and Apomixis. Certain plant species display aspects of fertilization-independent reproductive development, including apomictic generation of embryo and endosperm and development of the maternal seed coat and fruit (reviewed in ref. 33). The *fie* mutation reveals that *Arabidopsis*, a sexually reproducing plant, has the genetic potential for aspects of fertilization-independent reproductive development. It is not known whether the mechanism of fertilization-independent endosperm development conferred by the *fie* mutation is the same as autonomous endosperm formation observed in certain apomictic plant species. However, the fact that the *fie* phenotype is caused by a single genetic locus substantiates the view that the number of genetic differences between sexually and asexually reproducing plants is small (34).

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